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## P16INK4A and MIB-1 Expression in Preneoplasia and Neoplasia of Cervix

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### 1. Introduction

Cervical cancer is the third most common cancer and fourth most common cause of cancer related deaths in female population, accounting to approximately 453,300 cases per year and 275,100 deaths in the year 2008. According to the latest WHO global cancer statistics, the cumulative risk (%) (Age 0-74) of cervical carcinoma is 0.9 with age adjusted ratio of 9.0 (Jemal et al., 2011). In India, cervical cancer is the leading cancer among females between 15 and 44 years of age. Current estimates indicate that every year 132,082 women are diagnosed with cervical cancer and that 74,118 die from this disease in India alone (<http://www.who.int/hpv>). Having said this, however, no form of cancer better documents the remarkable effects of prevention, early diagnosis, and curative therapy on the mortality rate than the cancer of cervix. However, the still very high rate of cervical carcinoma in developing countries like India is because of lack of proper screening methods and lack of health infrastructure which allows for periodical and routine screening. Potential threat of cancer has reduced significantly in developed countries, due to Papanicolaou smear screening programs. Papanicolaou smears or commonly referred as Pap smears is a cost effective and reproducible screening technique for diagnosing precursor lesions of cervical carcinoma. However, Pap test gives significant false positive (30%) (Sherman et al., 1994) and false negative (15-50%) results due to subjective test criteria (Arbyn et al., 2008). Apart from the Pap smear screening test, histopathological diagnosis of cervical intraepithelial neoplasias (CINs) and cervical carcinoma is considered as the age old “gold standard” method of diagnosis of cervical neoplasms. However this can also be biased by interobserver variability as reported before (Stoler & Schiffman, 2001). These factors limit, present screening programs and histopathological examination and emphasizes the need for the identification of specific biomarkers for dysplastic epithelial cells to aid in primary screening and lesion diagnosis.

### 2. Cervical Intraepithelial Neoplasia (CIN)

Invasive squamous cell carcinoma of cervix is preceded by precancerous changes in the cervical epithelium which can be identified histologically. These precancerous lesions are usually described as Cervical Intraepithelial Neoplasia (Buckley et al., 1982). Papanicolaou classification, using the terms '**atypical cells with abnormal features**' has been adhered to, until recently by some cytologists and gynaecologists. In 1953 Reagan *et al.* proposed the

term “**dysplasia**” to replace atypical metaplasia and atypical hyperplasia. Ritton and Christopherson defined the normal and abnormal cells of cervical and vaginal smears in the WHO International classification (1973). The conventional histological terminology of mild, moderate and severe dysplasia and carcinoma in situ was used as well as atypical metaplasia. The British Society for clinical cytology’s first Working party on terminology recommended the term “**dyskaryosis**”, originally coined by Papanicolaou and translated from the Greek meaning “abnormal nucleus”, to describe cells from preinvasive and invasive cancer (Spriggs et al., 1978). In 1986, in a further review, dyskaryosis remained the recommended term, but it was classified as *mild*, *moderate* and *severe*.

The 1988 Bethesda System for reporting Cervical/Vaginal Cytologic Diagnoses was published by a Workshop of North American Experts convened by the Division of Cancer Prevention and Control of the National Cancer Institute to review existing terminology and to recommend effective methods of reporting. It agreed that the Papanicolaou classification was no longer appropriate and proposed the **Bethesda System**, which recommends three essential components of a cervical or vaginal smear report. It includes a new term, **Squamous intraepithelial neoplasia** (SIL) which is divided into two grades, **low grade SIL** (cells from HPV and CIN-I) and **high grade SIL** (cells from CIN II and CIN III) (Broder et al., 1991). A Bethesda workshop was held in 2001 with further modifications. The descriptions of cytological appearances of the cells of precancerous conditions of the cervix are best understood in relation to the well defined three histological grades of CIN (Solomon et al., 2002).

## 2.1 Low grade Squamous Intraepithelial Lesion (LSIL)

In LSIL the cells are mature squamous cells, they retain their polygonal shape and for the most part retain their normal size with a peripheral rim of dense cytoplasm. The nuclei are enlarged at least 3-4 times that of the normal intermediate cell nucleus, however, when HPV changes are evident, the cells may be smaller (almost parakeratotic) and the nuclei may also be smaller and somewhat pyknotic appearing with binucleation and/or multinucleation. These pyknotic nuclei also exhibit abnormal features such as hyperchromasia, increased size from that of the normal superficial squamous cell and a slight variation in shape and size. The chromatin appears finely to coarsely granular and is evenly distributed. It is important to stress that an interpretation of LSIL/HPV requires both clear-cut cytoplasmic cavitations accompanied by the abnormal nuclear morphology described above.

### Differential diagnoses

#### Reactive

The cells appear single or in sheets, like LSIL, however unlike LSIL, where only mature squamous cells are affected, in reactive types, all the cells may be affected. The nuclei, may be enlarged from 1.5 to 2 times; bi or multinucleation may be present and the nuclear membrane appear smooth. The chromatin is finely granular, evenly distributed and hyperchromasia may not be evident. The nucleoli are uniform and may be multiple in numbers. Peri-nuclear halo is often present, small and multiple vacuoles may be evident due to degeneration

### Reparative changes

The cells appear in flat sheets or groups. Predominantly, endocervical or metaplastic cells are affected. The nuclear size may be variable, ranging from slight to marked enlargement. The nuclei may be bi or multinucleated, and the nuclear membranes appear smooth. The chromatin is finely granular and evenly distributed and hyperchromasia may not be evident. The nucleoli are small to conspicuous and often multiple. The cytoplasm appears vacuolated.

## 2.2 High grade Intraepithelial Lesion (HSIL)

The criteria for HSIL on the ThinPrep® Pap Test are as follows: The single, most important criterion for HSIL is the presence of asymmetrical 3-D nuclear structural abnormalities. This is a concept that must be clearly understood in order to master the interpretation of HSIL. There will be an abnormality in the structure of the dysplastic nucleus that can be thoroughly appreciated only by focusing up and down on the individual cell. A normal nucleus has a relatively round or ovoid shape and its surface is smooth. A dysplastic cell will have humps, bumps, corrugations, crevices, and strange protuberances. These very distinctive abnormalities are the essence of dysplasia, particularly HSIL. This is the very detail that is most often lost in conventional cytology due to the various artefacts of fixation and staining that limit the ability to interpret these conventional smears. These 3-D nuclear structural abnormalities are to be distinguished from simple, "irregular nuclear outlines" which will often be present as a two-dimensional phenomenon in benign cells on the ThinPrep® Pap Tests.

These 3-D structural abnormalities may not be present in every dysplastic cell on the slide, but they will be obvious in at least some cells somewhere on the slides. Obviously, the ability to see "into" the nucleus of a cell is going to be directly related to the quality of staining. Also, these 3-D structural defects should be asymmetrical, as opposed to nuclear grooves or simple creases that involve the full breadth of the nucleus occasionally creating a difficult "look-alike". The presence of these exaggerated nuclear 3-D abnormalities establishes the diagnosis of HSIL.

Apart from above the N/C ratio is the most reliable indicator of degree (moderate, severe, CIS). With an increasing degree of dysplasia, there is a predictable increasing N/C ratio. This abnormal N/C ratio can be considered a major criterion for the diagnosis of HSIL. However, there are rare exceptions, and ultimately the diagnosis must be made on nuclear changes alone.

Gland neck involvement in HSIL has a distinct appearance on the ThinPrep Pap Test and can be differentiated from lesions of glandular origin. SIL in glands presents predominantly in sheets with increased depth of focus. The cytoplasm is finely vacuolated which initially may give the impression of a glandular process, but on closer inspection these sheets exhibit no glandular differentiation such as basal nuclei, crowded columnar formations, pseudostratification, nor feathered edges or rosettes. These sheets of cells can be deceptively flat, but the nuclei retain the same qualities of SIL that are described above. Because these cells are in sheets and usually are small with no other "clues" of HSIL - only subtle 3-D deformities, they can be the most difficult to identify and evaluate. An important factor in determining whether or not these cells are squamous or glandular in origin is the company they keep. Most of the time these cells will be accompanied by definitely dysplastic squamous epithelial cells.

### 2.3 Atypical Squamous Cells of Undetermined Significance (ASCUS)

ASCUS in the reproductive woman is defined by a number of criteria. The principal one is nuclear size using either an intermediate squamous cell nucleus or a mature metaplastic squamous cell nucleus as the reference standard. An ASCUS nucleus is 2.5 to 3 times the size of an intermediate cell nucleus or 1.5 times the size of a mature metaplastic cell nucleus. Hyperchromasia is commonly present, nucleoli are not prominent. These nuclear features are most important in diagnosing ASCUS.

### 3. Etiology of CIN and cervical carcinoma

There are various etiological factors leading to CIN and eventually to cervical carcinoma. Active and passive smoking (Brinton et al., 1986), dietary deficiencies (Butterworth et al., 1992), immunosuppression (Zur-Hausen, 1993) and sexually associated factors are a few to name. Among these, sexually associated factors (SAF) are the most important in pathogenesis of cervical carcinoma and CIN. Multiple sexual partners, early marriage (Munoz & Bosch, 1989), male sexual behaviour (Brinton et al., 1989), concurrent penile cancer in males (Li et al., 1982) and sexually transmitted diseases are strongly associated with the development of carcinoma. Viral infections with Herpes simplex virus- 2 (HSV-2) (Fenoglio et al., 1982) and Human Papilloma Virus (HPV) has been implicated and studied extensively in the etiopathogenesis of cervical carcinoma and CIN (Meisels et al., 1981)

#### 3.1 Role of HPV in etiology of cervical neoplasia

Innumerable experimental studies have provided strong evidence that HPV is the long sought venereal cause of cervical neoplasia. These viruses are double stranded DNA viruses (Baltimore Class I) and have been included traditionally in the Papovaviridae (Tomita et al., 1987). HPV is a double stranded Papilloma virus; 70 different types of which are known. Many different HPV types associated with cervical neoplasia have been discovered and around twenty types of HPV are commonly known to infect the human genital tract. These are HPV 6, 11, 16, 18, 30, 31, 33, 34, 35, 39, 40, 42, 51-58 (Crum et al., 1991). However they have been divided into high- and low-risk categories based on their association with invasive cervical carcinoma (Lorincz et al., 1992). Of this HPV 16, 18 and 31 are more commonly implicated in cervical carcinoma (Zur- Hausen, 1991). Experimental data indicate that viral E6 and E7 genes of high-risk HPV E7 protein specifically bind to and inactivate pRB (retinoblastoma gene product).

##### 3.1.1 Structure of HPV

HPV-DNA consists of three different regions: early region (ER), late region (LR) and upstream regulatory regions (URR). The ER is composed of seven genes, E1-E8, that encodes proteins which play a significant role in viral replication and have oncogenic properties. The LR is composed of two genes L1 and L2, which encodes proteins required for assembly of infectious viral particles. The URR is the regulatory region. In preneoplastic lesion like CINs the HPV DNA is not integrated in host DNA, rather it is found in circular or episomal form. Briefly, the episomal HPV produces mostly the E2 protein. The E2 protein encodes for a DNA-binding protein that binds to a specific nucleotide motif found in E6 and E7 region (Ham et al., 1991) (Ward et al., 1989). There therefore the E2 regulates the expression of E6



and E7, so that only low level of these proteins is produced. The episomal form integrates into the host's chromosome at E1/E2 region, typically causing "break" in this region, giving rise to uncontrolled production and expression of E6 and E7 proteins and their high levels are produced. Scheffner et al have shown previously that E6 protein forms a complex with p53 tumor-suppressor gene product, leading to the degradation of p53. Crook et al studied the expression of p53 in several HPV-positive or HPV-negative cell lines, and found that the HPV-negative cell lines had a mutation in a single nucleotide (a point mutation) in the p53 mRNA. These findings suggest that the loss of the wild type p53 protein activity is important in the development of a malignant lesion, and this could be mediated either by point mutation or by the binding of HPV E6 protein to p53. The HPV E7 protein binds to retinoblastoma tumor-suppressor gene product and inactivates the p Rb (Scheffner et al., 1992). The degradation of p53 and functional inactivation of p Rb leads to cell cycle disruption and increased proliferation, ultimately giving rise to carcinoma.

### 3.1.2 HPV detection techniques

Detection of HPV DNA in CIN and cervical carcinoma is the most popular and well investigated biomarker in management of cervical neoplasia. Various techniques are used to detect DNA. These are:

1. Immunocytochemistry
2. Dot Blot assays
3. Southern Blot
4. In situ Hybridization
5. Hybrid Capture™ 2 (HC2) assay
6. Polymerase chain reaction (PCR) techniques
7. HPV genotyping
8. Immunocytochemical detection of L1 capsid protein.

Among the above mentioned In situ hybridization. HC2 assays and PCR are most commonly used methods to detect HPV. Currently according to the updated guidelines published by American Society for Colposcopy and Cervical Pathology (ASCCP), the "HPV testing" refers only to Hybrid Capture 2 test for high-risk types (HPV16 and 18) (Wright et al., 2007). The HC2 test is the only test presently approved by U. S. Food and Drug Administration.

## 4. Biomarkers in cervical neoplasia

Cytomorphological interpretation of Pap stained cervical smears is the mainstay of cytological evaluation of the human cervix. A wide array of potential biomarkers is being evaluated for the diagnostic usefulness of cervical cancer and its precursors. One of the needs to identify biomarkers in cervical neoplasia is to distinguish CIN from other non neoplastic cervical lesions, so as to prevent under treatment (Al Nafussi et al., 1990) or overtreatment (Creagh et al., 1995). Second purpose is, that since CIN is a dynamic process (not a static process), that can progress or regress, the conventional haematoxylin and eosin (H&E), gives a false impression of a static process. These points emphasize the need to identify and discover new markers that can aid in distinguishing CIN from other benign conditions and establish it as a dynamic process. Since HPV, disrupts the normal cell cycle,

leading to cell death, a number of genes/ proteins are deregulated, thereby such genes/proteins can be used as surrogate diagnostic markers. In the past few years number of genes/proteins has been implicated as suitable biomarkers for cervical neoplasia. Two markers that have shown a potential in this direction are p16 INK4A and MIB1. p16 is a tumor suppressor protein, that is expressed in dysplastic cervical epithelial cells only, while MIB-1 is a marker of active dividing cells (basal and parabasal cells), normally not shed in cervical smears. Therefore presence of p16 and MIB-1 positivity in cervical Pap smear is marker of cervical dyskaryosis. Due to these reasons, p16 and MIB-1 have emerged as the most robust, stable and markers with strong predictive value.

#### 4.1 P16INK4A

P16INK4A (inhibitor of kinase 4A), is a tumor suppressor protein and inhibitor of cyclin-dependant kinase 4 and 6 (CDK 4 and 6). The phosphorylation of pRB (retinoblastoma protein) is a molecular "ON-OFF" switch for the cell cycle. In the hypophosphorylated form, pRB binds to transcription factors (p 16) responsible for cell cycle progression. P16 inhibits the cyclin-dependant kinases and thereby prevents the phosphorylation of RB, keeping it in the hypophosphorylated form, i.e. its active form. However, in HPV infection, the viral gene E7 binds to RB protein and functionally inactivates it. This results in accumulation of p16 protein because, normally, RB inhibits the transcription of p16 (Keating, 2001; Klaes, 2001; Sano, 1998). Because this protein is not expressed in the normal cervical epithelium, p16 overexpression allows to specifically identify dysplastic lesions and will reduce interobserver disagreement of conventional histological or cytological tests.

##### 4.1.1 P16INK4A as a diagnostic biomarker in cervical neoplasia

p16 INK4a is a tumor suppressor protein (cyclin dependant kinase inhibitor) which is known to play a critical role as a negative regulator of cell cycle progression and differentiation by controlling the activity of tumor suppressor protein pRb. We performed a study on the role of p16 and MIB-1 in cervical intraepithelial neoplasia. Our hypothesis was that normal cervical epithelium does not express p16 INK 4a and MIB-1 and there is upregulation of these biomarkers in CINs and cervical carcinomas. We evaluated p16 and MIB-1 in 63 cervical biopsies and corresponding Pap smears. p16INK 4A immunostaining was done using Mouse monoclonal antibody RTU-p16-432 (Novocastra, Lab. Ltd., Newcastle, Tyne, NE 128EW, UK, in all study groups. Immunopositive was considered when there was Either diffuse, strong nuclear and cytoplasmic staining, or focal moderate to weak nuclear staining of tumor cells. p16 INK4A immunohistochemistry revealed that there was a significant over expression and upregulation in different groups and as we move from normal cervical epithelia to dysplasia of varying severity to carcinoma, the p16 positivity was increased. p16 INK4A over expression was seen in all CIN I lesions (15/15), all CIN II lesions (15/15), all CIN III lesions (3/3) and all cases of carcinoma cervix (15/15) of tissue biopsies. In Pap smears p16 positivity was seen in CIN I/LSIL (8/10), CIN II/HSIL (5/5), CIN III/HSIL (3/3) and Ca cervix (15/15). No detectable p16 expression was observed in normal cervical epithelium in both pap smears and tissue biopsies. This was found to be statistically significant finding on making a comparison between control versus different groups ( $p < 0.05$ ). However, on making an inter group comparison this was found to be statistically insignificant ( $p > 0.05$ ). p16 basically is a nuclear protein hence immunohistochemistry should show nuclear staining. However in dysplasia

both nuclear and cytoplasmic staining with p16 is observed possibly because of post transcriptional modification or overproduction of p16 protein forcing its transfer into the cytoplasm (Murphy *et al.*, 2002). In our study it was seen that p16 over-expression was restricted to CIN I, II, III and carcinoma cervix and increased in the same order. Therefore, p16 immunostaining allowed precise identification of even small CIN or cervical cancer lesions in biopsy sections and Pap smears and helped to reduce interobserver variation and also reduce false positive and false negative interpretation and thereby significantly improve cervical cancer and precancer detection (Srivastava, 2010). In our study p16 positivity was 15 of 15 (100%) in invasive carcinoma cervix and it was seen that with increasing severity of CINs, p16 positivity increased. Similar results were seen in a study by Murphy *et al.* who observed 100% p16 positivity in invasive SCC and significant linear relation ( $p < 0.0001$ ) between p16 staining and increasing grades of squamous dysplasia (Murphy *et al.*, 2002, 2005). We also observed that two Pap smears with LSIL showed negative p16 staining whereas it was positive in corresponding CIN lesion of their tissue section. p16 may be rarely negative in cervical dyskaryosis that may have important implications for the use of p16 staining as a standalone test and support the use of combination of markers of cervical dyskaryosis (Murphy *et al.* 2005). However in our study we did not find any dysplasia negative for p16 in tissues biopsies. p16 staining in LSIL was found to be negative in 20% of Pap smears, which could possibly be due to the technical error as their corresponding sections showed consistent positivity.

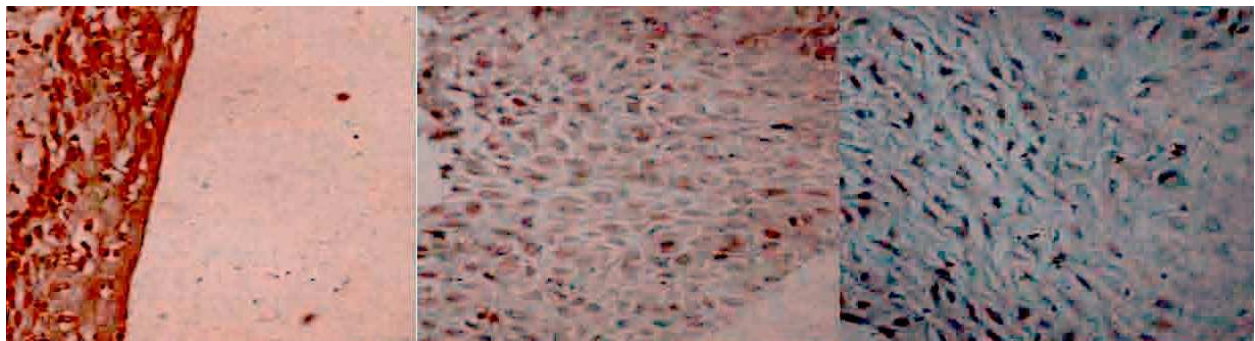


Fig. 1. Images of p16 immunostaining in Cervix tissue biopsy in CIN I, CIN III, and Carcinoma cervix (40X)

A study conducted by Trigler *et al.*, in 2004, confirmed that the proportion of pRb positive cells was relatively decreased in premalignant and malignant lesions of the squamous and endocervical mucosa and showed a generally inverse correlation with the expression of p16 at the tissue level. This feedback loop is bypassed via viral E7 interaction and inactivation with pRb, causing p16 to be up regulated which can be detected immunohistochemically. p16 could therefore have a clinical utility as a biomarker because it is a measure of HPV gene expression and activity, rather than solely a detector of viral presence (Stanley 2002). Negri *et al.* in 2003 conducted a study to determine whether immunostaining of p16 is useful in detecting adenocarcinomas of cervix and its precursors in histologic & cytologic routine specimens. A total of 45 patients with glandular lesions including 18 cases of adenocarcinoma in situ (AIS), adenocarcinoma (n=8), endocervical glandular atypia (n=4) and reactive (n=15) lesions were identified. Furthermore, immunocytochemical analysis was performed on 10 Thin Prep Smears with abnormal glandular cells. P16 was detected immunohistochemically on all 26 cases of AIS and adenocarcinoma (100%). Also the



immunocytochemical detection on thin prep specimens evidenced a strong expression of p16 in neoplastic endocervical cells. Prior to this study Mc Cluggage *et al.* (2003) investigated the value of p16 immunoreactivity in the distinction between endometrial and endocervical adenocarcinomas. Cases included in this study were endometrial adenocarcinomas of endometrioid type (n=29), and cervical adenocarcinomas of endocervical type (n=23). Twenty-two of 23 endocervical adenocarcinomas showed 100% positive tumor cells. The maximum number of endometrial adenocarcinomas, 9 of 29 showed 21-50% positive tumor cells. They concluded that diffuse strong positivity with p16 suggested an endocervical rather than an endometrial origin of an adenocarcinoma. Endometrial adenocarcinomas are usually positive, but positivity is generally focal and involves less than 50% of cells. Therefore, when there is a morphological doubt then this antibody may be of value as part of a panel for ascertaining the origin of an adenocarcinoma.

Determining the origin of uterine adenocarcinomas can be difficult in biopsy and curettage specimens because the morphologic spectrum of endocervical (ECA) and endometrial adenocarcinomas (EMA) overlap. Ansari Lari *et al.* in 2004 evaluated the utility of immunohistochemistry for P16 in the distinction of ECAs and EMAs. p16 expression was assessed in 24 unequivocal EMA's and 19 unequivocal ECA's and correlated with HPV DNA detected by ISH and PCR. p16 expression was moderate – strong and diffuse in 18 and weak and diffuse in 1 ECA. Fourteen of these were positive for HPV DNA. EMA's displayed weaker staining with patchy distribution and none contained HPV DNA by ISH. Compared with HPV DNA detected by *in situ* hybridization, p16 immunohistochemistry appears to be more sensitive and easier to perform, method for distinguishing ECAs from EMAs. It can be used to assist in the classification of lower uterine segment/endocervical adenocarcinomas of equivocal origin and should be evaluated for its utility in the prospective classification of uterine adenocarcinoma in curettage specimens prior to hysterectomy. Giovanni Negri *et al.* in 2004 evaluated the immunohistochemical expression of p16 as a marker of progression risk in low-grade dysplastic lesions of the cervix uteri. IHC was performed on 32 CIN-I with proven spontaneous regression of lesion in follow up (group A), 31 with progression to CIN-3 (group B) and 33 that were randomly chosen irrespective of the natural history of lesion (Group C). A diffuse staining was detected in 43.8% of CIN-I of group A, 74.2% of group B and 56.3% of group C. Overall 71.4% and 37.8% of p16 negative and diffusely positive CIN-I had regressed, at follow up, where as 26.6% and 62.2% negative and diffusely CIN-I were progressed to CIN-III ( $p<0.05$ ). Although p16 may be expressed in low grade squamous lesion that undergoes spontaneous regression, in this study CIN-I cases with diffuse p16 staining had a significantly higher tendency to progress to a high grade lesion than p16 negative cases. Therefore, p16 may have the potential to support the interpretation of low grade dysplastic lesions of the cervix uteri. Sahebali *et al.* in 2004 examined the potential of p16 INK4a as a potential biomarker for cervical lesions in a study of liquid based cervical cytology. HPV DNA testing by MY09/MY11 consensus PCR and type specific PCRs and p16INK4a immunocytochemistry on a series of 291 patients selected from routine screening was done. Comparison of the number of p16 immunoreactive cells / 1000 cells exhibited a significantly higher mean count ( $8.80\pm1.13$ ) than other cytological groups. The mean count of LSIL ( $1.09\pm0.18$ ) was significantly higher than other negative groups. Atypical squamous cells, cannot exclude high grade squamous intraepithelial lesion (ASC-H) and HSIL combined showed a significantly higher mean count ( $6.46\pm1.17$ ) than negative

ASC, ASCUS and LSIL. Thus p16 immunocytochemistry can be used as an adjunct to LBC in cervical screening, because it has a good diagnostic accuracy to discriminate HSIL and ASC-H (atypical squamous cells – cannot exclude HSIL) from other lesions. It could be used as a surrogate marker of high risk infections.

Kalof *et al.* in 2005 studied the correlation between p16 immunoexpression, grades of CIN and HPV type in 44 cervical biopsies classified as CIN-I and CIN-II/III. In 22 of 25 CIN-I lesions, p16 immuno expression was confined to lower half of the epithelium with sporadic to focal staining in 11 of 25 cases. In CIN-II/III, 15 of 17 showed diffuse 2/3 to full thickness staining of the epithelial. hr HPV were found in 20 CIN-I lesions and 17 CIN-II/III lesions. Punctate signals were detected in 3 of hr HPV positive CIN-I lesions and 17 of 17 CIN-II/III lesions. They found that p16 immunoexpression and the presence of punctate signal on HPV in situ hybridization correlated with degree of cervical neoplasia ( $p < 0.001$ ). Thus both increased p16 immunoexpression and punctate signal correlates with CIN-II/III grade, supporting the use of either, or both tests to confirm CIN-II/III.

P16 can be used as a diagnostic marker along with other well known markers implicated in cervical neoplasia. N Murphy *et al.* in 2005 analysed and compared expression patterns of three potential biomarkers p16, CDC6 and MCM5 and evaluated their use as predictive biomarkers in squamous and glandular pre invasive neoplasia. 20 normal cervical biopsies, in addition to 38 CIN-I, 33 CIN-II, 46 CIN-III, 10 SCC, 19 CGIN and 10 adenocarcinoma were included in the study.. In all normal cases cervical epithelia were not stained. Dysplastic epithelial cells showed p16 staining in 100% of CIN-I, CIN-II, CIN-III, SCC and, adenocarcinoma. Simple linear regression analysis revealed a highly significant linear relation between p16 and increasing grades of squamous dysplasia. Among 3 markers p16 was the most reliable marker of cervical dysplasia. It marked all grades of squamous and glandular lesions of the cervix, and its expression was closely associated with high risk HPV infection. However, the failure of p16 to mark an isolated CIN-III case and staining of glandular mimics as tubo endometrioid metaplasia, may limit its use as a standalone test of cervical dysplasia. Thus a combination of dysplastic markers is suggested in difficult cases.

#### 4.2 MIB-1 as a proliferation marker in cervical neoplasia

MIB-1 (Molecular Immunology Borstel) is an important diagnostic marker for CIN. Gerdes *et al.* in 1990 demonstrated that MIB-1 antibody detects Ki-67 antigen (in paraffin embedded biopsies) in G<sub>1</sub>, S, G<sub>2</sub> and M phase but is absent in G<sub>0</sub> phase. Baak *et al* formulated a “Stratification Index” (SI, which indicates, how high Ki-67 positive nuclei are located in the epithelium; the higher the SI, the higher the CIN grade) and the number of Ki-67 nuclei per 100  $\mu$ m basal membrane (the more Ki-67 nuclei, the higher the grade) to distinguish the three CIN grades at the same time.

Ki-67 is an antigen expressed in proliferating cells (Brown, 2002) that can be detected in formalin fixed tissues using the MIB-1 antibody (Cattoretti *et al.*, 1992). MIB-1 is an important immunocytochemical marker to assess the proliferation and has been suggested as a sensitive biological indicator of progression in CIN lesions by Van Hoven *et al.*, 1997. We performed MIB-1 Immunohistochemistry along with p16 in 63 biopsies of cervical neoplasia and their corresponding Pap smears. MIB-I immunohistochemistry revealed that

there was a significant over expression of MIB-1 in different groups and as we move from normal cervical epithelia to varying severity of CINs to carcinoma, the MIB-1 positivity increased. This was found to be statistically significant finding on making a comparison between control versus different groups ( $p<0.05$ ). However, on making an intergroup comparison this was found to be statistically insignificant ( $p>0.05$ ). MIB-1 antibody detects Ki-67 Antigen in G1, S, G2 and M phase but is absent in G0 phase. Therefore, this antibody may be a useful marker of proliferative activity of premalignant and malignant lesions of cervix. In our study we found that as we move from normal to carcinoma group via the varying degrees of CIN, labeling index of positively stained nuclei increased with the severity of CIN to carcinoma group. Review of published literature showed that Goel *et al.* (2005) have also observed similar results. Proliferative index was significantly increased in the carcinoma group in comparison with dysplasia. They showed the following trend for both MIB-1 and PCNA.

Normal < LSIL < HSIL < Carcinoma

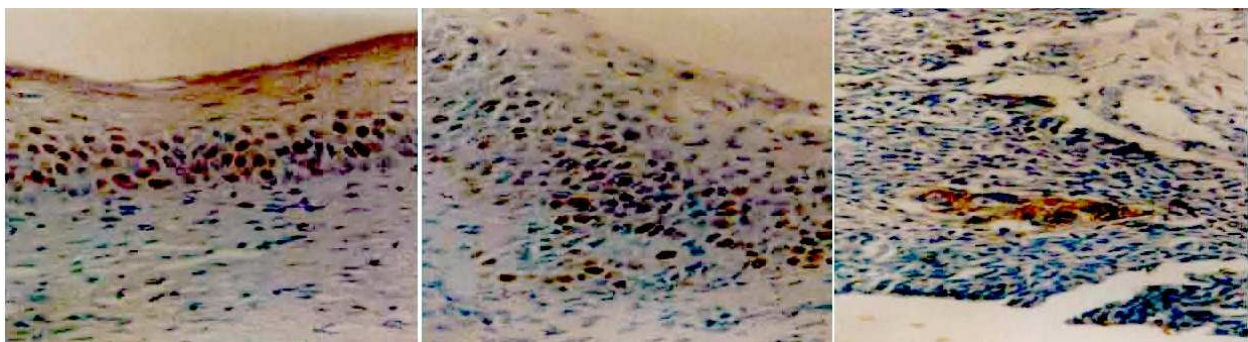


Fig. 2. Images of MIB-1 immunostaining in Cervix tissue biopsz in CIN I, CIN II, and Carcinoma cervix (40X)

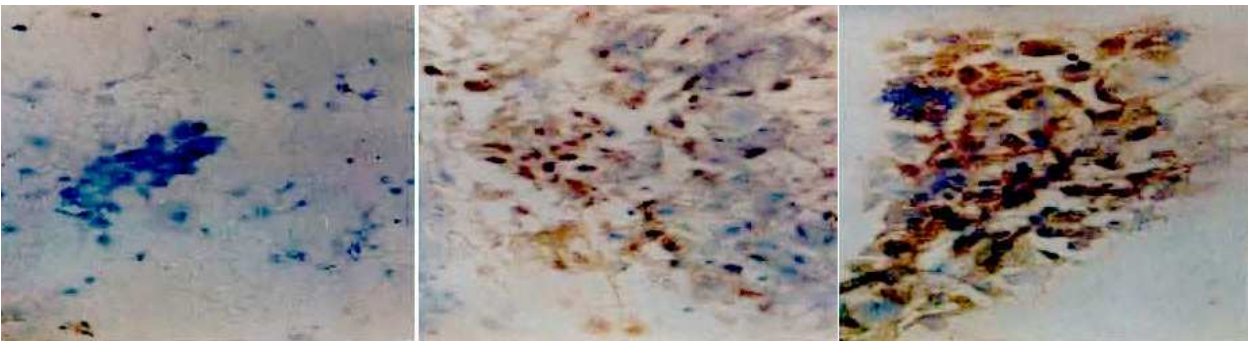


Fig. 3. Images of MIB-1 staining in Pap smears (40X)

In a study by Garzetti *et al.* (1996), MIB-1 immunostaining as an index of cellular proliferations in CIN and micro invasive carcinoma was analysed, with the aim to identify a relationship with the degree of dysplastic lesion and the risk of neoplastic progression. 41 cases of CIN, 23 cases of cervical condyloma, 22 of squamous metaplasia and 10 with micro invasive carcinoma were selected. It was shown that a) positive MIB-1 immunostaining



increased progressively from squamous metaplasia to CIN and micro invasive carcinoma, ( $p < 0.001$ ) suggesting that neoplastic proliferation is associated with dysfunctional proliferation of cervical epithelium. b) Considering only CINs the MIB 1 index showed a significant increase with respect to CIN degrees, ( $p < 0.0001$ ). c) That there is a significant correlation between the MIB-1 index and CIN degree but not with respect to HPV DNA presence and d) that MIB-1 immunostaining might be useful for a clinical evaluation of mild and moderate dysplastic lesions. Gorstein et al in 2000 found that in cervical intraepithelial lesions associated with infection by HPV types 16 and 18, the expression of Ki 67 is greater than in lesions unrelated to viral presence.

Prior studies have suggested that Ki-67 (MIB-1) and p16 expression may be preferentially expressed in cervical neoplasia. However, a study conducted by Keating *et al.* in 2001, examined and compared the distribution of staining of these antigens in normal and reactive epithelial changes, diagnostically challenging cases (atypical metaplasia and atrophy) SIL, and high and low risk HPV, type specific SIL. Overall, a histologic diagnosis of SIL correlated strongly with these biomarkers used. Positive scores for Ki-67 and p16 were seen in 68.4% and 100% of LSILs and 94.7% and 100% of HSILs respectively.

P16 INK4a and Ki-67 biomarkers have been evaluated in conventional histopathological sections and more recently on Pap smears. However Akpolat *et al.* in 2004 evaluated the utility of P16 INK4a and Ki-67 staining on cell blocks prepared from residual thin layer cervicovaginal material. Results of cytological based thin prep Pap test were SCC (n=3), HSIL (n=27), LSIL (n=20), ASCUS (n=11), negative for malignancy (n=24). Results of cell blocks preparation were, SCC (n=2), HSIL (n=20), LSIL (n=30), negative for malignancy (n=32). In 62 cases (73%) the diagnosis made using cell blocks were in agreement with thin pap smears. The results indicate that cell blocks represent an additional reliable diagnostic tool in the evaluation of cervical samples<sup>52</sup>. Chisa Aoyama *et al.* in 2005 conducted a study to determine that histologic and immunohistochemical characteristics are useful for distinguishing neoplastic and non-neoplastic lesions. They classified atypical squamous lesion (ASL - a histologic diagnosis of unclear significance in the uterine cervix) (n=37) into neoplastic (n=19) and non-neoplastic (n=18) groups. They chose 7 histologic and IHC indicators to classify ASL. Mitosis, vertical nuclear growth pattern, no perinuclear halo, indistinct cytoplasmic border, primitive cells in the upper third of the squamous layer, p16+ cells in the upper 2/3 of squamous layer and Ki67 positive cells in upper 2/3 of squamous layer were significant indicators for neoplastic ASLs (5 or more of these 7 indicators). Out of 19 ASL, 16 had 5 or more of these indicators. Majority of non-neoplastic ASLs, 16/18 had 2 or fewer indicators.

In a study done by Goel *et al* in 2005, 49 adequate pap smears were stained for MIB-1 and PCNA. Out of 49 cases, 40 cases showed positive immunostaining with MIB-1 and PCNA. Proliferative labelling index of MIB-1 increased with ascending grades of CIN lesions to carcinoma. The highest proliferative index for MIB-1 was observed for the carcinoma group (PCNA-LI  $39.200 \pm 1.865$ ; MIB-1 LI  $35.300 \pm 1.888$ ). A significant positive correlation between ascending grades of SIL and LI of markers ( $r = 0.87$  for MIB-1 and  $r = 0.88$  for PCNA) was seen. This suggests that MIB-1 can be used as an adjunct to cytomorphological interpretation of conventional cervical Pap smear.

	Authors	Year	Number of cases	Results
1	Valasoulis et al	2011	95/LSIL smears	SS=41%;SP=86% PPV=62%;NPV=72%
2	Mendez et al		67/abnormal cytology	35.8% cases positive by p16 and associated with HPV
3	Samir et al	2011	188/pap smears	P16 correlates with increasing CIN grade
4	Balan et al	2010	20/LSIL, HSIL	P16 positive in 68% LSIL;84% CIN2;100% CIN3
5	Schmidt et al	2011	776/ ASCUS, LSIL P16/ Ki-67 dual stain cytology	SS=92.2% ASCUS; 94.2% LSIL SP=80.6% ASCUS;68% LSIL
6	Petry et al	2011	425/pap negative; HPV positive P16/ Ki-67 dual stain cytology	25.4% positive; SS=91.9% for CIN2; 96.4% for CIN3 SP=82.1% for CIN2; 76.9%for CIN3
7	Alameda et al	2011	109/ frozen sections of ASCUS	SS=82.3%; SP=100%; NPV=94.5%; PPV=100% for HSIL
8	Passamonti et al	2011	91/ ASCUS;60 LSIL;36 ASCH;59 HSIL	46% ASCUS;53% LSIL
9	Srivastava et al	2010	63 /cervical biopsy and pap smears	P16 positive in increasing grades of CIN
10	Bolanca et al	2010	81/ cervical smears	33.3% of HPV positive cases showed p16 positivity
11	Oberg et al	2010	64/ LBC	86% agreement between ProEx C and p16



	Authors	Year	Number of cases	Results
12	Sung et al	2010	105/ ASC-H and ASC-US	P16 correlated significantly with SIL in ASC-H smears
13	Yu et al	2010	63 /cell blocks	HPV L1 and p16 expression increased with severity of cervical lesions
14.	Adamopoulou et al	2009	62 /abnormal pap smears and biopsies	P53, p16 and Bcl-2; SS=83.3%; SP=65.4%
15	Kurshumliu et al	2009	312/ pap smears	36.2% positive for p16
16	Haidopoulos et al	2009	62/abnormal pap smears	SS=100%; SP=76%; PPV=61%; NPV==100%
18	Dray <i>et al.</i>	2005	18/Biopsies 188/Thin pap smears	p16 +ve in HSIL, LSIL. -ve in inflammatory and reactive changes
20	Pientong <i>et al.</i>	2004	165/ pap smear 165/LBC	p16 +ve in 0/30, 21/40, 19/35, 30/30, 30/30 in normal, ASCUS, LSIL, HSIL, Ca.
21	Zielenskii <i>et al.</i>	2002	142/Biopsies of glandular neoplasia	All ACIS and ADCA were HPV positive therefore hr HPV testing is must in cervical cancer screening programme
22	Agoff <i>et al.</i>	2003	569/Biopsies	p16 and Ki67 correlated with cervical neoplasia and HPV
23	Klaes <i>et al.</i>	2002	194/Cervical biopsies	p16 improves the interobserver agreement in diagnosis of CIN

Table 1. Review of literature

LSIL, low grade squamous intraepithelial lesion; SS, sensitivity; SP, specificity; NPV, negative predictive value; PPV, positive predictive value; HPV, human Papilloma virus; CIN, cervical intraepithelial neoplasia; HSIL, high grade squamous intraepithelial lesion; ASCUS, atypical squamous cell of unknown significance; ASCH, atypical squamous cell cannot exclude high grade squamous intraepithelial neoplasia; LBC, liquid base cytology; ACIS, adenocarcinoma in situ; ADCA, adenocarcinoma

## 5. Conclusion

In a tropical country like India, any perimenopausal women presenting in gynaecological out patient department with any complaint is subjected to a single Pap smear test. However single Pap test is subject to suboptimal sensitivity limited reproducibility and many a times with high rate of false positive and false negative along with equivocal results. To compensate for the aforementioned deficiencies, a screening programme with repeated testing, and follow up of positive cases is warranted. Moreover, colposcopic performed biopsy is directed in any suspicious appearing acetowhite area. This subjects the patient to unnecessary surgical intervention. Therefore, additional diagnostic and prognostic markers for detection of cervical cancers precursors are required which could save the patients from surgical intervention and high screening cost associated with repeated testing.

Also, biomarkers that can help in screening, detection, diagnosis of the disease as well as predict the prognosis can aid the clinicians in correct management of the patients. P16 and MIB-1 are two such candidate markers that fit well in the above mentioned criteria. Through our study we have thus concluded that for LSIL, (because the sensitivity of the p16 marker is 80%), the marker should be evaluated together with MIB-1 or HPV test. For HSIL, the sensitivity and specificity of the p16 marker is 100% and thus it can be used as a stand-alone test. We also recommend that with a careful interpretation of immunostaining with morphological characteristic in the conventional Pap smears, the immunostaining with p16 and MIB-1 markers may be a diagnostic adjunct, reducing the need of tissue biopsy. This is simple, reliable and easily applicable in routine cytosmears. Having said this, there is still need of validation of these markers in a larger cohort and targeted population.

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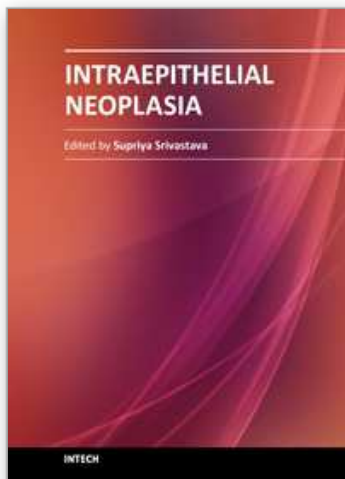
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The book "Intraepithelial neoplasia" is till date the most comprehensive book dedicated entirely to preinvasive lesions of the human body. Created and published with an aim of helping clinicians to not only diagnose but also understand the etiopathogenesis of the precursor lesions, the book also attempts to identify its molecular and genetic mechanisms. All of the chapters contain a considerable amount of new information, with an updated bibliographical list as well as the latest WHO classification of intraepithelial lesions that has been included wherever needed. The text has been updated according to the latest technical advances. This book can be described as concise, informative, logical and useful at all levels discussing thoroughly the invaluable role of molecular diagnostics and genetic mechanisms of the intraepithelial lesions. To make the materials easily digestible, the book is illustrated with colorful images.

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